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INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

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Luana Moro

**EFEITOS COMPORTAMENTAIS DA PREGABALINA E GABA-CAPRÓICO EM  
PEIXE-ZEBRA ADULTO FRENTE AO TESTE DE *NOVEL TANK* E A CRISES  
EPILÉPTICAS INDUZIDAS POR PENTILENOTETRAZOL**

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Trabalho de conclusão de curso de graduação  
apresentado ao Instituto de Ciências Básicas da  
Saúde da Universidade Federal do Rio Grande do  
Sul, como requisito parcial para obtenção do título  
de Bacharela em Biomedicina.

Orientador: Prof. Dr. Diogo Losch de Oliveira

Co-orientador: Me. Ben Hur Marins Mussilini

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Aprovado em: \_\_\_\_ de \_\_\_\_\_ de \_\_\_\_.

**BANCA EXAMINADORA**

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Nome do professor - instituição

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Nome do professor - instituição

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Nome do professor - instituição (orientador)

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## 1. Lista de abreviaturas

AMPA	$\alpha$ -amino-3-hidroxil-5-metil-4-isoxazol-propionato
DMSO	Dimetilsulfóxido
GABA	Ácido gamma-aminobutírico
GABA <sub>A</sub>	Receptor GABAérgico ionotrópico
KA	Cainato
NMDA	N-metil D-Aspartato
PTZ	Pentilenotetrazol
SNC	Sistema nervoso central
SV2A	Synaptic vesicle glycoprotein 2A (Proteína da vesícula sináptica 2A)



## 2. Resumo

A epilepsia é uma desordem neurológica que afeta aproximadamente 50 milhões de pessoas no mundo. Muitos pacientes diagnosticados com epilepsia sofrem de comorbidades psiquiátricas, como por exemplo a ansiedade, e sabe-se que isso pode levar a diminuição na qualidade de vida e na resposta aos fármacos antiepilépticos. Nós modificamos a estrutura química da pregabalina que deu origem a um derivado chamado GABA-capróico. Nosso objetivo foi analisar os efeitos comportamentais causados pelo tratamento de pregabalina e GABA-capróico no modelo de peixe-zebra adulto expostos a um aparato que representa novidade e também contra crises epiléticas induzidas por pentileotetrazol (PTZ). Os animais foram pré-tratados com pregabalina (10, 20, 60 mM) ou GABA-capróico (60 mM) dissolvidos em DMSO 1%) via injeção intraperitoneal. Após 30 min, os animais foram transferidos para o aquário trapezoidal (*novel tank*) a fim de avaliar parâmetros de locomoção e exploração dos animais e em aquários retangulares com solução de pentilenotetrazol na concentração de 10 mM para analisar o comportamento frente a crises epiléticas. A locomoção geral dos animais não apresentou alterações significativas. Os parâmetros exploratórios analisados não mostraram diferença estatística, porém, os animais demonstram uma tendência de aumentar o tempo de permanência no topo do aparato e diminuir o tempo de latência para a subida ao topo quando comparado com grupo controle. Os dados sobre crises epiléticas demonstraram que a pregabalina é capaz de diminuir a severidade das crises na dose de 60 mM. A pregabalina na dose de 20 mM e o GABA-caproico 60 mM tendem a diminuir a intensidade da crise e a latência para a crise epilética clônica, mas não apresentam diferença estatística significativa. Concluindo, a pregabalina e seu derivado tendem a apresentem efeitos tipo ansiolíticos no peixe-zebra, não causando efeitos adversos na locomoção. Além disso, a pregabalina é capaz de reduzir a intensidade das crises epiléticas induzidas por PTZ na concentração de 60 mM.

### 3 Introdução compreensiva

#### 3.1 Epilepsia

A epilepsia é definida como uma desordem cerebral na qual os neurônios encontram-se excessivamente ativos de forma sincronizada, e essa atividade anormal pode gerar alterações neurobiológicas e cognitivas, bem como transtornos psicológicos e problemas sociais (Fisher *et al.*, 2014). Essa desordem está entre as mais comuns, afetando pessoas de todas as idades. Dados epidemiológicos estimam que 50 milhões de indivíduos no mundo tenham epilepsia (Banerjee *et al.*, 2009). Diferentes históricos de pacientes podem levar ao diagnóstico de epilepsia. Segundo definições recentes da Liga Internacional Contra Epilepsia (ILAE), um indivíduo pode ser considerado epilético após a ocorrência de uma única crise não provocada, se esse indivíduo apresentar alto risco de ter outra crise similar dentro de 10 anos, como no caso de indivíduos que sofreram alguma injúria cerebral, por exemplo, infecções, traumas ou acidente vascular cerebral (Fisher *et al.*, 2014). Também pode ser caracterizado com essa condição o indivíduo que apresentar crises epiléticas recorrentes (duas ou mais) com 24h de diferença, cuja causa não seja identificada, ou pacientes com diagnóstico de síndrome epilética (Fisher *et al.*, 2005; Banerjee *et al.*, 2009).

Na última década, muitos pacientes com epilepsia foram diagnosticados também com variadas comorbidades psiquiátricas. As comorbidades mais reportadas em estudos são desordens de humor, psicoses, neuroses e desordem de personalidade. Dentre essas, as desordens de humor, particularmente a depressão e a ansiedade, são as mais frequentes desordens relatadas (Tellez-Zenteno *et al.*, 2007). A depressão em pacientes com epilepsia vem sendo muito estudada e, segundo a literatura, esses indivíduos apresentam diminuição na qualidade de vida, aumento do risco de suicídio e uma piora na resposta a tratamentos farmacológicos; portanto, esses pacientes devem ser devidamente diagnosticados e tratados (Kanner, 2011). Em contraste, a ansiedade é um tema pouco abordado, mas capaz de causar impactos negativos tanto quanto a depressão na vida desses pacientes.

### 3.2 Fármacos antiepilépticos

Ao final da década de 80, o tratamento para epilepsia era restrito aos medicamentos fenobarbital, fenitoína, carbamazepina e ácido valproico, que até hoje estão entre os mais receitados mundialmente (Bialer, 2002). Apartir da década de 90, deu-se início a revolução no tratamento dessa desordem. Levando em consideração o grande número de pacientes epiléticos que não respondem aos tratamentos farmacológicos existentes (aproximadamente 30%) e a lista de efeitos colaterais causados por esses medicamentos (Tabela 1), notou-se a necessidade de buscar novas terapias com maior eficácia e tolerância (Brodie e Dichter, 1996; Loscher, 2011).

**Tabela 1: Efeitos colaterais importantes dos fármacos antiepilético** (Brodie e Dichter, 1996).

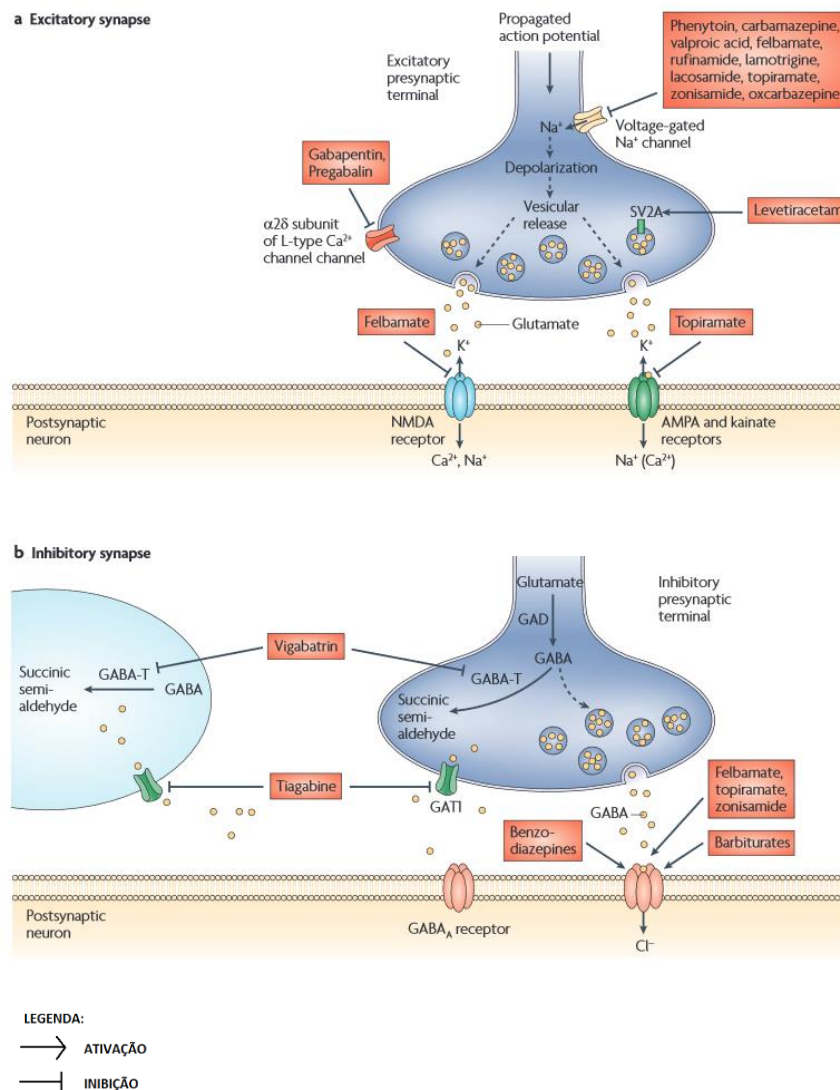
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<b>Fármaco</b>	<b>Efeito colateral dose-dependente</b>
<b>Fenobarbital</b>	Fadiga, desatenção, depressão; específico para crianças: insônia, distração, hipercinesia, irritabilidade
<b>Fenitoína</b>	Nistagmo, ataxia, náusea, vômito, depressão, sonolência, aumento paradoxal na crise epilética, anemia megaloblástica
<b>Carbamazepina</b>	Visão dupla, tontura, dor de cabeça, náusea, sonolência, neutropenia, hiponatremia
<b>Ácido valproico</b>	Tremor, ganho de peso, dispepsia, náusea, vômito, alopecia, edema periférico

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Atualmente existem diversos grupos de fármacos antiepiléticos, por exemplo: (1) fármacos que modulam predominantemente canais  $\text{Na}^+$  (e  $\text{Ca}^{2+}$ ) como a fenitoína, carbamazepina, oxcarbamazepina, lamotrigina e a zonisamida; (2) fármacos que modulam predominantemente canais de  $\text{Ca}^{2+}$ : etosuximida; (3) fármacos que modulam o sistema GABAérgico: benzodiazepínicos, vigabatrina, tiagabina; (4) ações mistas:

valproato (diminuição da ação de canais de  $\text{Na}^+$  dependentes de voltagem e inibição da degradação do GABA aumentando seus níveis na fenda sináptica, entre outras), felbamato (bloqueia canais de  $\text{Na}^+$  e o receptor NMDA, modulador do receptor  $\text{GABA}_A$ ), topiramato (bloqueia canais de  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , potencia a ação do GABA, bloqueia receptores AMPA/Ka e aumenta correntes de potássio), fenobarbital (aumento da afinidade do receptor  $\text{GABA}_A$  pelo neurotransmissor GABA, ação sobre o glutamato e bloqueio de canais de  $\text{Na}^+$ ); (5) fármacos recentes com novos alvos, com mecanismos pouco conhecidos: gabapentina (bloqueio de canais  $\text{Ca}^{2+}$ ), pregabalina (canais de  $\text{Ca}^{2+}$ ), levetiracetam (se liga a SV2A que pode estar envolvida na liberação de neurotransmissores), lacosamida (aumenta a inativação lenta dos canais de  $\text{Na}^+$ ) e retigabina (Czapinski *et al.*, 2005; Bialer e White, 2010; Loscher, 2011) (**Figura 1**).



**Figura 1. Mecanismo de ação sobre as sinapses excitatórias e inibitórias proposto para os fármacos antiepilépticos atuais** (Czapinski *et al.*, 2005). Num balanço global, fármacos antiepilépticos atuam diminuindo o sistema excitatório e aumentando o sistema inibitório dos neurônios.

Na busca por novos fármacos, podemos encontrar compostos que foram descobertos de forma empírica e possuem estrutura química completamente nova, e outros que derivam de fármacos antiepilépticos pré-existentes. O objetivo de desenvolver fármacos com base em algum pré-existente, é, além de melhorar a eficácia, segurança e tolerância do fármaco, aumentar a biodisponibilidade cerebral, eliminar a toxicidade dos metabólitos do composto original e evitar os elementos estruturais que tornam a molécula original possivelmente teratogênica (Bialer e White, 2010). Mesmo com todos os novos fármacos introduzidos no mercado e seus novos alvos, os problemas enfrentados anteriormente não desapareceram por completo; no entanto, pode-se dizer que em termos de segurança, tolerância e farmacocinética melhorias consideráveis foram alcançadas (Perucca *et al.*, 2007).

A escolha do fármaco antiepiléptico deve ser feita com cuidado, após análise dos sintomas do paciente. O diagnóstico correto do tipo de epilepsia é essencial para a escolha da terapia mais eficaz (Brodie e Dichter, 1996). A Liga Internacional Contra Epilepsia propôs um guia para o tratamento de pacientes epilépticos de acordo com o tipo de crise e idade, e quais seriam as melhores opções de substituição de terapia caso o fármaco de primeira escolha não seja eficaz para aquele indivíduo, baseado em estudos da literatura que indicam a capacidade de cada fármaco antiepiléptico como monoterapia (Glauser *et al.*, 2006).

### **3.2.1 Pregabalina**

A pregabalina é um fármaco desenvolvido recentemente e destaca-se na clínica com seu espectro de indicações. Utilizada no tratamento de crises parciais epilépticas, dor neuropática e transtornos de ansiedade generalizada, a pregabalina tornou-se amplamente prescrita (Kustermann *et al.*, 2014). Apesar de ser análoga estrutural do GABA, a pregabalina exerce sua ação ao se ligar à subunidade  $\alpha 2$ -delta de canais de

cálcio dependentes de voltagem na região pré-sináptica dos neurônios (Lotarski *et al.*, 2014). A ligação a esse sítio atenua a despolarização induzida pelo influxo de  $\text{Ca}^{2+}$  nos terminais nervosos, levando à diminuição na liberação de diversos neurotransmissores, como o glutamato, noradrenalina e substância P (Arroyo *et al.*, 2004). Além disso, apresenta caráter hidrofílico, mas atravessa a barreira hematoencefálica (Dooley *et al.*, 2000).

Tanto em crianças quando adultos, a pregabalina é utilizada em pacientes como tratamento adjunto em casos de crises parciais (com ou sem generalização secundária) refratários ao tratamento clássico, e apresenta perfil de tolerância e segurança aceitáveis (Arroyo *et al.*, 2004; Mann *et al.*, 2014). Os relatos mais comuns de efeitos adversos foram de sonolência e tontura (em adultos e crianças), visão dupla e aumento de peso (em adultos), vômitos, falta de coordenação motora, constipação, irritabilidade, febre (em crianças) (Arroyo *et al.*, 2004; Mann *et al.*, 2014).

Alguns estudos recentes apontam efeitos adversos mais graves relacionados ao uso da pregabalina. Dois casos de depressão acompanhados de ideação e prática suicida já foram relatados na clínica, alertando sobre esse possível efeito adverso, mesmo que raro, causado pelo uso de pregabalina (Kustermann *et al.*, 2014). Além disso, em ratos, já se tem estudos demonstrando um possível efeito teratogênico do medicamento mesmo em doses baixas (Etemad *et al.*, 2013). Portanto, apesar dos estudos clínicos sugerirem a pregabalina como uma das terapias adjuntas mais promissoras devido a sua alta eficiência, segurança e perfil farmacocinético vantajoso, estudos mais aprofundados ainda são necessários (Arroyo *et al.*, 2004).

### **3.3 Modelo animal peixe-zebra**

O peixe-zebra (*Danio rerio*), chamado também de *zebrafish* ou paulistinha, é um pequeno teleósteo (3-4 cm) da família dos Cyprinidae. No final da década de 60, a biologia do peixe-zebra começou a ser abordada por George Streisinger, que dentre diversas espécies de peixes tropicais estudadas, destacou o peixe-zebra por apresentar vantagens de criação, reprodução e de manipulação gênica (Grunwald e Eisen, 2002). Esse modelo animal tornou-se popular rapidamente, e, como uma espécie de vertebrado, o zebrafish tem uma alta homologia genética e fisiológica quando comparado a

humanos, além de possuir neuroanatomia e neurofisiologia conservada (Panula *et al.*, 2006; Alsop e Vijayan, 2008; Panula *et al.*, 2010).

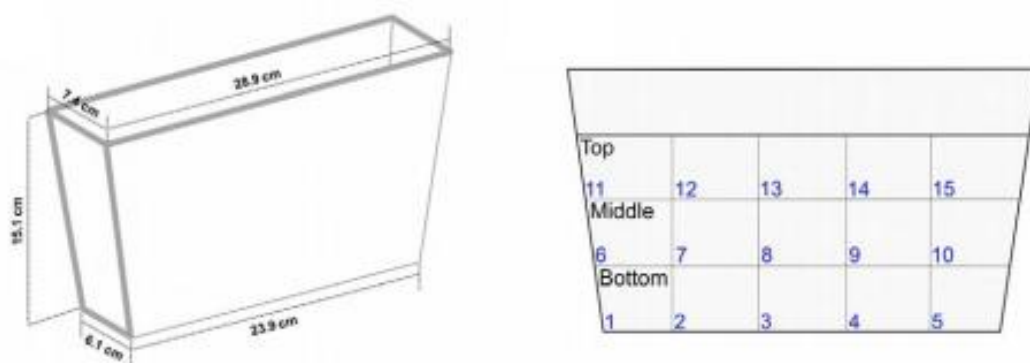
Desde então, no cenário de pesquisas, o peixe-zebra vem ganhando cada vez mais espaço e aplicações, e dentre essas se destaca a utilização do modelo para varreduras farmacológicas. O peixe-zebra possui a vantagem de ter reprodução que chega a gerar centenas de ovos a cada fecundação e, devido ao seu rápido desenvolvimento embrionário (em 24h apresenta estrutura básica corporal), em pouco tempo já é possível realizar ensaios biológicos em larga escala (Goldsmith, 2004). Além disso, o pequeno espaço requerido para manutenção dos animais e a quantidade reduzida de fármacos gastos são pontos de interesse quando se procura testar diversas moléculas com grande amostra experimental (Lieschke e Currie, 2007). Portanto, ao comparar modelos animais utilizados para reproduzir doenças humanas, percebemos a vantagem no modelo do peixe-zebra que apesar da similaridade anatômica conservada entre vertebrados, as condições experimentais são realizadas na escala de modelos invertebrados (Kalueff *et al.*, 2015).

### 3.4 Teste de open/novel tank

Semelhante ao teste de *open field* realizado em roedores, o teste *novel tank*, também conhecido como *open tank*, é utilizado para avaliar o comportamento do peixe-zebra. Esse teste fornece uma avaliação espaço-temporal do perfil locomotor e exploratório do animal sujeito a um ambiente que representa novidade (Rosemberg *et al.*, 2011). Através desse aparato experimental trapezoidal e da sua divisão virtual em diferentes zonas (Figura 2), é possível inferir, por exemplo, o perfil geral de locomoção através de parâmetros como distância total percorrida, tempo móvel, tempo imóvel, imobilidade e velocidade média e máxima. O repertório de resultados sobre a locomoção geral dos animais pode ser correlacionado com a presença ou ausência de efeitos colaterais em modelos animais comumente encontrados em diversos fármacos, como letargia, episódios de imobilidade e comportamento de *burst* (Ibrahim *et al.*, 2014).

O perfil exploratório horizontal e vertical pode ser analisado através de diversas medidas como latência para entrada em áreas, tempo em cada área e transições entre as áreas delimitadas virtualmente (Rosemberg *et al.*, 2011). Quando expostos ao aparato

*novel tank*, o peixe-zebra tende a explorar pouco o aparato, demorar mais para atingir a área do topo, e entrar menos vezes na área do topo (Levin et al., 2007). Esse perfil exploratório que o animal apresenta frente a um ambiente novo é reconhecido como um comportamento do tipo ansiogêncio (Levin et al. 2007). A correlação entre exploração do aparato e o comportamento tipo ansiolítico dos animais foi proposto em roedores, e mais recentemente para o modelo de peixe-zebra, baseando-se no fato de que animais tratados com fármacos ansiolíticos clássicos geram um aumento no comportamento exploratório dos animais pelas áreas delimitadas do aparato, menor latência para entrada na área do topo, tempo de permanência na área do topo aumentada, aumento no número de transições na área do topo (Cryan e Holmes, 2005; Stewart *et al.*, 2012).



**Figura 2. *Novel tank*.** Tanque trapezoidal com divisões virtuais utilizadas para avaliar a atividade natatória do peixe-zebra, com divisão de três áreas verticais (topo, meio e fundo) e quinze áreas horizontais.

### 3.5 Modelos de indução de crises epilépticas em peixe-zebra

O peixe-zebra apresenta um grande potencial como modelo animal para estudos sobre a epilepsia, tanto com animais geneticamente modificados para essa patologia ou com modelo de crises epilépticas agudas provocadas (Grone e Baraban, 2015). Para que um modelo animal de uma patologia ou de uma desordem seja considerado válido, ele precisa reproduzir características essenciais das condições humanas (Sarkisian, 2001), no caso das epilepsias, por exemplo, apresentar descargas elétricas cerebrais anormais e comportamentos estereotipados (Baraban *et al.*, 2005).



Para gerar mutações no peixe-zebra, a técnica de injeção de morfolinós é realizada para gerar *gene knockdown*, que são oligonucleotídeos modificados que se ligam e bloqueiam a tradução do mRNA endógeno, diminuindo a expressão do gene alvo relacionado à epilepsia (Teng *et al.*, 2010; Suls *et al.*, 2013). Outro método mais eficaz utilizado é o uso do plasmídeo CRISPR\*Cas9 gerando deleções (*knockout*) ou inserções (*knock-in*) em alelos específicos do DNA do peixe-zebra (Grone e Baraban, 2015). Esses animais geneticamente modificados apresentam crises epiléticas espontâneas.

Quanto a modelos de indução de crises aguda, temos o modelo de hipertermia e modelos químicos. No modelo de hipertermia, a crise epilética é induzida pelo aumento controlado na temperatura do banho em que se encontram as larvas de peixe-zebra, que produz registros eletrofisiológicos semelhantes aos dos animais com crises epiléticas induzidas pelo agente químico pentilenotetrazol (Hunt *et al.*, 2012). Quanto aos modelos químicos, temos os clássicos agentes pró-convulsivos: pentilenotetrazol (PTZ), antagonista não-competitivo dos receptores GABA<sub>A</sub>, e ácido cáínico (KA), agonista de receptores glutamatérgicos. O modelo químico de PTZ vem sendo amplamente utilizado para descoberta de novos fármacos antiepiléticos (Loscher, 2011). Em um sistema equilibrado, o neurotransmissor GABA ativa o receptor GABA<sub>A</sub>, levando ao influxo de Cl<sup>-</sup> através desse canal iônico e à hiperpolarização neuronal. Na presença do agente pró-convulsivo, ocorre redução no influxo de Cl<sup>-</sup>, diminuindo a inibição neuronal, gerando um desequilíbrio entre o sistema nervoso excitatório e inibitório, o que desencadeia a crise epilética (Hansen *et al.*, 2004). É bem estabelecido que o agente químico PTZ provoca, tanto em peixe-zebra larvas quanto em adultos, um aumento no sistema alterações nos registros eletroencefalográficos e padrões comportamentais estereotipados que já estão bem descritos na literatura (Baraban *et al.*, 2005; Pineda *et al.*, 2011; Mussulini *et al.*, 2013). Em relação ao ácido cáínico, as alterações elétricas cerebrais devido a exposição das larvas de peixe-zebra a esse agente químico também estão descritas, bem como o comportamento estereotipado apresentado tanto em larvas quanto em adultos (Kim *et al.*, 2010; Alfaro *et al.*, 2011; Menezes *et al.*, 2014).

#### **4. OBJETIVO GERAL**

O presente projeto visa avaliar os possíveis efeitos promovidos pela pregabalina e seu análogo sintético, GABA-capróico, frente ao teste baseado no paradigma de novidade e também frente à indução de crises epiléticas induzidas por pentilenotetrazol no modelo de peixe-zebra adulto.

##### **4.1 Objetivos específicos**

4.1.1 Buscar a concentração ideal de aplicação da pregabalina e seus derivado sintético no peixe-zebra a ser utilizada como pré-tratamento em crises epiléticas induzidas pelo PTZ;

4.1.2 Investigar os efeitos comportamentais (atividade locomotora e exploratória) induzidos pela pregabalina e GABA-capróico isoladamente no peixe-zebra durante 6 min após passados 30 minutos da injeção do pré-tratamento;

4.1.3 Investigar os efeitos comportamentais do pré-tratamento com pregabalina e GABA-capróico nas concentrações ideais no comportamento do peixe-zebra frente a crises epiléticas induzidas por pentilenotetrazol durante 20 minutos após 30 min da administração do pré-tratamento;

4.1.4 Avaliar a taxa de mortalidade dos animais pré-tratados com as moléculas durante 5 dias posteriores à indução de crise epilética.

## **5. Trabalho experimental na forma de artigo científico**

Este trabalho foi escrito em formato de artigo científico, seguindo as normas para submissão de artigo da revista Epilepsy Research, as quais estão contidas no anexo 1.

**Behavioral effects of pregabalin and GABA-caproic on adult zebrafish in the novelty paradigm and pentylenetetrazole induced epileptic seizure.**

Luana Moro<sup>1</sup>, Caroline Da Ros Montes D'Oca<sup>2</sup>, Ben Hur Marins Mussulini<sup>1</sup>, Marcelo G. Montes D'Oca<sup>3</sup>, Dennis Russowsky<sup>2</sup>, Diogo Losch de Oliveira<sup>1</sup>.

<sup>1</sup>Programa de pós-graduação em Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

<sup>2</sup>Programa de pós-graduação em Química, Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, 91501-970, Porto Alegre, RS, Brazil.

<sup>3</sup>Programa de pós-graduação em Química Tecnológica e Ambiental, Escola de Química e Alimentos, Universidade Federal do Rio Grande, Av. Italia, Km 08, s/ n°, 96203-900, Rio Grande, RS, Brazil.

Corresponding author: Luana Moro Tel.: + 55 51 33085555;

E-mail address: [luanamoro222@gmail.com](mailto:luanamoro222@gmail.com)

### **HIGHLIGHTS**

- Pregabalin treatment reduced seizure intensity induced by pentylenetetrazole in adult zebrafish.
- GABA-caproic showed a tendency to decrease seizure intensity induced by pentylenetetrazole.
- Pregabalin and GABA-caproic tend to display anxiolytic profile in the novel tank test and both did not modify locomotor parameters in adult zebrafish.

## ABSTRACT

Epilepsy is a common brain disorder affecting approximately 50 million people worldwide. Several studies have assessed psychiatric comorbidities in epilepsy, such as anxiety. Despite AED therapies progression, new compounds have been studied. Pregabalin, a recent drug developed to treat epilepsy, was chemically modified generating a derivative called GABA-caproic. We evaluate the behavioral effect of pregabalin and GABA-caproic in adult zebrafish model in the novelty test paradigm and also against epileptic seizures induced by pentylenetetrazole. Fish were pretreated with an intraperitoneal (i.p) injection of pregabalin (10, 20, 60 mM) or GABA-caproic (60 mM) dissolved in DMSO 1% ( $n= 8$  in each group). After 30 min of pretreatment, animals were transferred to the trapezoidal apparatus (novel tank) to assess locomotion and exploratory behavior or to rectangular apparatus filled with pentylenetetrazole 10 mM to analyze seizure behavior. General locomotion did not show modification between pretreated animals and control group. Parameters analyzed for exploratory behavior did not show statistical difference; although, animals exhibit a tendency to spend more time in the top area and decrease latency to the top compared to control. Seizure parameters analyzed demonstrated that only pregabalin at 60 mM concentration is able to reduce seizure intensity. Pregabalin 20 mM and GABA-caproic 60 mM tend to decrease seizure intensity and latency to clonic seizure-like behavior onset in lower levels. In conclusion, pregabalin and GABA-caproic tend to present anxiolytic-like effects in adult zebrafish, but it did not cause locomotor side effects. Pregabalin exhibit anticonvulsant effect at 60 mM in PTZ-induced seizure model in adult zebrafish, while pregabalin 20 mM and GABA-caproic 60 mM were able to prevent epileptic seizure in a certain degree.

**Key words:** Zebrafish, Pregabalin, Anxiety, Epilepsy, GABA-caproic.

## 1. Introduction

Epilepsy is characterized as a brain disorder in which neurons exhibit an abnormal excessive or synchronized activity, and this abnormal activity can generate neurobiological and cognitive modifications, as well as psychological and social disorders (Fisher et al., 2005). Epilepsy is the most common disorder, affecting people of all ages, and epidemiologic data estimates that over 50 million people worldwide have a diagnosis of the disorder (Banerjee et al., 2009). Despite therapeutic advances, studies have shown that approximately 30% of patients diagnosed epileptic do not respond to the proposed pharmacological treatments (Brodie and Dichter, 1996). Moreover, in the last decades, many epileptic patients were diagnosed with psychiatric comorbidities (Gaitatzis et al., 2004). Among them, anxiety is one of the most common disorder related (Tellez-Zenteno et al., 2007).

In last decades, new drugs with anticonvulsant activity were successfully developed, and many compounds are resultant of structural modification of a pre-existing molecule. Despite AED therapies progression in the last few decades, new compounds have been studied in order to enhance treatment quality (Loscher, 2011). Recently, a gamma aminobutyric acid (GABA) structural analog, pregabalin, was accepted for refractory epilepsy treatment (Hamandi and Sander, 2006).

Although pregabalin and GABA share structural similarity, pregabalin binds to the auxiliary  $\alpha_2\delta$  subunit of voltage-sensitive calcium channels at presynaptic terminals (Dooley et al., 2000). Pregabalin is largely used in cases of partial epileptic seizures and generalized anxiety disorders. It showed to be efficient, well tolerated and with a safety acceptable profile as add-on treatment for partial seizures in adults and children and generalized anxiety disorder in adults (Alvarez et al., 2015; Arroyo et al., 2004; Mann et al., 2014). Although it displays high efficiency, studies suggest that pregabalin should not be prescribed in treatment of idiopathic generalized epilepsy (Hamandi and Sander, 2006). In addition, some recent studies in mice have been shown that pregabalin can be teratogenic even in lower doses (Etemad et al., 2013) and some severe adverse effects have been related, such as depression with suicidal attempt (Kustermann et al., 2014). At this point, it seems that more research about pregabalin effects is needed and

chemical modifications in pregabalin molecule seem to be an interesting goal to develop new compounds.

The development of new AED face a problem to be discovered by the same animal models (Loscher, 2011). It seems to be essential to improve preclinical development of AED and zebrafish emerges as an alternative approach for pharmacological screening in biomedical research. We chose to work with adult model instead of larval model due to its brain complexity, including neurotransmitter systems and blood-brain barrier well developed, which are extremely important in AED research (Ullmann et al., 2010). Furthermore, drugs with low aqueous solubility are difficult to tested in larval zebrafish because of precipitation and lack of body absorption, while in adult zebrafish another administration, such as i.p injection can be performed (Banote et al., 2013). Besides, these animal model fulfill some prerequisites for emergent animal model utilized when testing large number of investigational compounds, like handling and storage convenience and time- and cost-efficient (Loscher, 2011). These characteristics turn the zebrafish model attractive and it could be as an alternative for the homogeneity in AED discovery. Thus, to detect antiepileptic efficacy in drug development, pentylenetetrazole is a chemical model of induce epileptic seizure largely indicated and this model is well described in zebrafish (Baraban et al., 2005; Mussulini et al., 2013).

In accordance to literature, long alkyl chain derivative compounds have shown several biological activities, suggesting the potential application in the construction of new chemical entities or by incorporation in the molecular structure of commercial available drugs (D'Oca Cda et al., 2010; Rodrigues et al., 2013). Thus, pregabalin was chemically modified with an insertion of a fatty acid chain from caproic acid, generating a new derivative that we called GABA-caproic. Therefore, to assess the behavioral effects of GABA-caproic in zebrafish adult model, we tested the locomotor profile and anxiolytic-like behavior in the novel test paradigm and also we tested GABA-caproic as a pharmacological pretreatment against epileptic seizures induced by pentylenetetrazole. To accomplish this goal, we first needed to characterize the effect of pregabalin in zebrafish adult model.

## **2. Materials and Methods**

### **2.1. Ethics statement**



All procedures with animal subjects have been approved by the Ethics Committee for Use of Animals- CEUA from Universidade Federal do Rio Grande do Sul (protocol number 27006).

## 2.2. Reagents

Ethyl 3-aminobenzoate methanesulfonic acid salt, pentylenetetrazole and dimethyl sulfoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pregabalin and GABA-caproic were synthesized by Instituto de Química from Universidade Federal do Rio Grande do Sul, RS, Brazil. Their chemical structures were elucidated and patented (BR102013012934-A2).

## 2.3. Chemical

The compounds were synthesized in a four step strategy from isovaleral and caproic aldehydes (**Fig. 1**). First, the aldehydes were submitted to Knoevenagel condensation with Meldrum's acid (2) in the presence of piperidinium acetate to afford de alkylidene derivatives (3a-b) (Milite et al., 2011). The intermediates (3a-b) were converted to nitroderivatives (4a-b) by Michael addition of  $\text{CH}_3\text{NO}_2$ , that after opening Meldrum's portion and decarboxylation domino process lead to the nitroacid intermediates (5a-b) (Kimmel et al., 2012). Finally, the reduction of nitro group resulted in the Pregabalin (6) or GABA-caproic derivative (7) in global yields 60-65% respectively. The compounds were characterized by NMR  $^1\text{H}$  and  $^{13}\text{C}$ , IR and elemental analysis.

## 2.4. Animals

Adult male and female zebrafish (6-8 months-old) of heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). Fish were housed in 40-L aquariums (70-100 fish per aquarium) for at least 2 weeks prior to the experiments in order to acclimate to the animal facility. They were maintained on a constant 14/10-h light/dark cycle (lights on at 8:00 am). Water from all tanks was aerated, maintained with mechanical and chemical filtration at  $26 \pm 2^\circ\text{C}$  and water pH at 7.0-8.0. Animals were fed twice a day with commercial flake fish food (alcon, BASICH, Alcon®, Brazil) and twice a day with artemia cysts (3h

intervals). Animals used in this study were experimentally naive, healthy and free of any signs of disease. They were maintained according to the National Institute of Health Guide for Care and Use of Laboratory Animals (2011).

## **2.5. Treatments with pregabalin and GABA-caproic**

Animals were removed from their tanks, and individually transferred to beakers filled with 300 mL of home system water in which they were weighed. After, animals were anesthetized by immersion in tricaine solution 160 µg/ml (Alfaro et al., 2011) and in the absence of reflex response to slight mechanical pressure on the tail, fish were pretreated with an intraperitoneal (i.p) injection of pregabalin (10, 20, 60 mM) or GABA-caproic (60 mM) dissolved in DMSO 1% (Siebel et al., 2015). Control group received vehicle DMSO 1%. The volume injected was 10 µl/g of body weight. The concentrations of pregabalin tested in zebrafish were based on the dose capable to prevent seizure in 50% of mice (Lotarski et al., 2014).

Fish were subsequently transferred back to their individual beakers where they remained during 30 minutes. Fish were placed into the apparatus to run the experiments.

In order to perform experiments with similar conditions, all tests were conducted during the same period each day (8:30 am to 1:00 p.m.) and the animals were handled carefully to reduce stress and avoid interference in locomotor activity.

## **2.6. Behavioral analysis**

### **2.6.1. General locomotion and exploratory behavior**

After pretreatment with pregabalin (10, 20 and 60 mM) and GABA-caproic (60mM), fish ( $n=8$  in each group) were individually transferred to a trapezoidal tank (23 cm along the bottom x 28.3 cm at the top x 16 cm in height x 7 cm width), dimensions similar to those previously described, filled with 1.5L home system water called novel tank (Rosemberg et al., 2011). A webcam (HD Pro, C920–Logitech) was placed (40 cm) front of the novel tank to monitor locomotor activity during 6 minutes.

In order to ensure a uniform background for the video analysis and also prevent animals' distraction due to environmental factors, yellow sheets of paper were placed

around (10 cm near) novel tank. Furthermore, one 60-W light bulb was placed behind the yellow sheet to enhance contrast between background and fish and improve quality of the animal's tracking. The webcam was connected to a laptop and the video analyses were performed by ANY-maze® software (Stoelting, CO, USA) at the rate of 20 positions/second. General locomotion was analyzed by the total distance travelled and time immobile. Exploratory behavior was analyzed by time spent in the three horizontal areas, entries to the top area, and latency to enter the top area.

### **2.6.2 Pentylenetetrazole induced seizure**

Animals ( $n=8$  in each group) received pregabalin (20 and 60 mM) and GABA-caproic (60 mM) prior exposure to the proconvulsant agent pentylenetetrazole (PTZ).

To assess possible antiepileptic effect of pregabalin and GABA-caproic, fish were individually transferred to a rectangular tank (20 cm long x 13 cm high x 7 cm width) filled with 1L of PTZ at a concentration of 10 mM dissolved in water. Behavioral phenotype of the PTZ seizure model in adult zebrafish was evaluated by scores: (0) Short swim mainly in the bottom of the tank, (1) Increased swimming activity and high frequency of opercular movement, (2) Burst swimming, left and right movements, and erratic movements, (3) Circular movements, (4) Clonic seizure-like behavior (abnormal whole-body rhythmic muscular contraction), (5) Fall to the bottom of the tank, tonic seizure-like behavior (sinking to the bottom of the tank, loss of body posture, and principally by rigid extension of the body), (6) Death. All tank walls were coated with white cover, in order to avoid the reflex of the animal in the glass walls, and to standardize background for the video recording. A webcam (HD Pro, C920–Logitech) was placed 25 cm in front of the rectangular tank coupled to a laptop in order to record animal behavior during 20 minutes. All behavioral data were evaluated by 1 trained observer according to the behavioral characterization of PTZ induced seizure in adult zebrafish well established in the literature (Mussulini et al., 2013). After exposure to PTZ, mortality was assessed during 5 days.

### **2.7. Statistics**

Locomotion was expressed by mean $\pm$ S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. Entries to the top area were

expressed as median $\pm$ interquartile range and it was analyzed by Kruskal Wallis test. Non-parametric data of seizure scores were expressed as median $\pm$ interquartile range. Cumulative frequency was determined using the percentage of animal that reached each score across time for the respective treatment tested. The area under the curve (AUC) and latency were represented as mean $\pm$ S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. In all analyses, the significance level was taken as  $p \leq 0.05$ .

### 3. Results

Animals treated with pregabalin (6, 20 and 60 mM) did not show significant statistical differences compared to control group in general locomotor parameters measured, such as distance travelled (**Fig. 2A**) and time immobile (**Fig. 2B**). However, animals pretreated with pregabalin at concentration 6 mM have a tendency to spend more time immobile. As the highest concentration of pregabalin tested did not alter locomotor parameters, we selected 60 mM to test the same parameters with GABA-caproic (**Fig. 2A, 2B**) and the animals behave similar to control group.

Endpoint analysis used to measure potential anxiolytic-like effects of molecules were analyzed for pregabalin and GABA-caproic treatment. (**Fig. 3, 4**). Although no statistical differences were founded in time spent in each horizontal area of the apparatus, animals treated with pregabalin in highest concentrations (20 and 60 mM) and GABA-caproic (60 mM) exhibit a tendency to spend more time in the top area instead of bottom area (**Fig. 3 A, C**). We did not find differences statistically significant in the number of entries to the top area, although there is a tendency in animals pretreated with pregabalin 20 mM and GABA-caproic 60 mM to enter more times in the top area of the novel tank (**Fig. 4A**). The latency to enter top area seems to decrease in the groups treated with pregabalin and GABA-caproic at concentration of 60 mM (**Fig. 5A**).

After analyze pretreatments effects separately, we tested the effects of pregabalin (20 and 60 mM) and GABA-caproic (60 mM) as pretreatment in PTZ-induced seizure. All groups exhibited stereotyped movements when exposed to PTZ 10 mM, classified in different scores (**Fig. 6**). Control group (DMSO 1%) shown behavioral profile with

rapid score progression in first 5 min (**Fig. 6A**). Fish which received pregabalin 20 mM and GABA-caproic 60 mM exhibit seizure profile similar to control group, although both showed scores increasing slower during the first 5 min (**Fig. 6B, 6D**). Animals pretreated with pregabalin 60 mM showed remarkable difference during the first 5 min, exhibiting low score progression (**Fig. 6C**). In the last 15 min of PTZ immersion, animal of all groups displayed a stable similar seizure profile, predominantly clonic seizure-like behavior or fall to the bottom of the tank and loss of body posture with tonic seizure-like behavior, the highest scores in the behavioral characterization of PTZ seizures in adult zebrafish (**Fig. 6 A, B, C, D**).

In order to evaluate the seizure intensity across time in these three moments, we measured the area under score curve for each animal for each group. In the first interval (0-150 s), fish pretreated with pregabalin 60 mM exhibited lower seizure intensity when compared to control (one-way ANOVA,  $F_{[3,28]} = 3,26$ ,  $p < 0.05$ ; Bonferroni test  $p < 0.05$  (**Fig. 6E**). The other two groups, pregabalin 20 mM and GABA-caproic 60 mM did not show statistical difference, although both seizure intensity are lower when compared to control (**Fig. 6E**). In the second interval (150 – 300 s), no statistical difference was found, but the pretreatment with the highest concentration of pregabalin and GABA-caproic tend to decrease seizure intensity (**Fig. 6F**). In the last interval (300–1200 s), as the animals showed almost the same behavior in seizure score curve, seizure intensity are very similar and it did not showed significant statistic difference (**Fig. 6G**).

Cumulative scores frequency shows that control group which exhibit the most intense seizure curve and intensity, rapidly change from score 2 to score 4, with just few animals presenting score 3 (25%) (**Fig. 7A**). As the seizure intensity in pretreated animals decreases, more animals reach score 3, pregabalin 20 mM (50%), pregabalin 60 mM (87.5%) and GABA-caproic (62.5%) (**Fig. 7B, C, D**). Also, 100% of the control group and pregabalin 20 mM reached score 4 before 300 s and almost all, 75% and 50%, respectively, reached score 5 (**Fig. 7A,B**). In contrast, this stereotype score is showed by 100% of pregabalin 60 mM and GABA-caproic 60 mM after 300 s and just 12.5% and 25% of the animals exhibited score 5 (**Fig. 7C, D**). An end point measure for AEDs, the latency to clonic seizure behavior, was measured for each group pretreated during PTZ exposure. Only pregabalin at the concentration 60 mM showed statistical

difference (one-way ANOVA  $F_{[3,28]} = 6.832$ ,  $p < 0.001$ ; Bonferroni test  $p < 0.05$  (**Fig. 8A**).

Mortality was assessed during 5 days after PTZ exposure. Only animals exposed to highest concentration of pregabalin (60 mM/kg) and GABA-caproic (60 mM) died (score 6), 12.5% and 25%, respectively.

#### 4. Discussion

Zebrafish became a very time-efficient model used in biomedical drugs discovery and it is an emerging model in order to understand complex brain disorders (Kalueff et al., 2014). To develop new therapies based on pregabalin structure in the zebrafish model, a description of pregabalin is necessary, and there are no previously evidence of pregabalin effects in adult zebrafish in the literature. In our study, we showed the effects of anticonvulsant pretreatment on chemoconvulsant-induced seizure-like behavior in adult zebrafish an anxiolytic-like effects by behavioral assessment.

Similar to the open field apparatus used for rodents, the open tank for fish is used to assess locomotor and exploratory behavior in zebrafish (Rosemberg et al., 2011). Locomotion endpoint analysis can be used to measure potential toxic effects of the compounds. In this study, we measured the swimming performance of fish by the distance travelled and time immobile. Although no statistical difference was found, animals pretreated with pregabalin (20 and 60 mM) tend to enhance swimming activity and reduce time immobile when compared to control, while animals treated with 60 mM GABA-caproic showed similar behavior profile to control group. Hyperlocomotion has been reported in zebrafish in response to MK-801 treatment, which showed anxiolytic-like effects at the novel tank test (Swain et al. 2004; Stewart et al., 2012). These results show that the pretreatments did not evoke side effects such as lethargy or increase in time immobile (Ibrahim et al., 2014).

Correlation between exploratory behavior with anxiolytic-like behavior was proposed for rodents and zebrafish (Cryan and Holmes, 2005; Stewart et al., 2012). Our results about exploratory profile showed that animals which received pregabalin and GABA-caproic tend to increase exploratory behavior in the open tank, spending more time in the top area, increasing entries to the top area and reducing latency to enter the

top when compared to control when compared to control group. Zebrafish naturally exhibit a reduced exploratory profile and preference to the bottom area in response to exposure to an apparatus which represent novelty and could induce anxiogenic-like behavior, so this results could indicate reduced anxiety levels in the fish as it was previously reported (Levin et al., 2007). We believe that absence of statistical significance is due to small number of animal in each group, and also some animals inside each group exhibit very distinct behavior compared to majority; therefore, it is important to perform an additional open tank experiment increasing number of animals to find true effects of both compounds.

As it would be valuable to develop therapies with both anxiolytic and antiepileptic action, we performed the behavioral sequence score profile of seizure induced by PTZ as described in the literature (Mussulini et al., 2013). The score-system during 20 min analysis showed a control group pattern similar to the one found in literature, with rapid score progression in first 5 min, and animals presenting high seizure scores in the last 15 min, leading to a high intensity seizure profile. Lower seizure intensity found in group pretreated with pregabalin 60 mM in first 150 s is a sign found in fish pretreated by immersion in anticonvulsant solution (Mussulini et al., 2013). Literature data about absence or presence of circular movements (score 3) is controversial, but in our experiments manifestation of low scores, such as score 3, become more prominent in all groups which received pretreatment, and it seems that proportional increase as seizure intensity decrease (Banote et al., 2013).

Moreover, classical parameter of latency to clonic seizure-like behavior largely used in development of new AED was measured for all pretreatments (Bachiega et al., 2008; Ben-Menachem et al., 2010). Statistical significance was found for group pretreated with pregabalin at concentration of 60 mM, while pregabalin at 20 mM and GABA-caproic 60 mM showed, despite no statistical significance, increase in latency to score 4 onset. Percentage mortality found in groups pretreated with pregabalin and GABA-caproic 60 mM suggest that may be compounds are toxic in a certain level, but more studies about toxic mechanism are needed. In contrast, our control mortality did not follow mortality rate of 33% in control group. With all data together, we can infer that pregabalin reduce seizure severity in a concentration dependent way.

## **Conclusion**

Summarizing, pregabalin and GABA-caproic tend to present anxyolytic-like effects in zebrafish adult, but did not cause locomotor side effects. Pregabalin exhibit anticonvulsant effect at 60 mM in PTZ-induced seizure model in adult zebrafish, while pregabalin 20 mM and GABA-caproic 60 mM were able to prevent epileptic seizure in a certain degree. These findings turn adult zebrafish into an animal model able to be used in AED mechanism comprehension and development using pregabalin as pharmacological base.

## **5. Conflict of interest**

None of the authors have any conflict of interest to disclose.



**Fig. 1. Synthesis of tested compounds Pregabalin (6) and GABA-Caproic (7).** Aldehydes (1a, 1b) submitted to Knoevenagel condensation with Meldrum's acid (2) in the presence of piperidinium acetate to afford de alkylidene derivatives (3a-b) and then they were converted to nitroderivatives (4a-b) by Michael addition of  $\text{CH}_3\text{NO}_2$ , that after opening Meldrum's portion and decarboxylation domino process lead to the nitroacidintermediates (5a-b). The reduction of nitro group resulted in the pregabalin (6) or GABA-caproic derivative (7).

**Fig. 2. Endpoint behaviors used to assess locomotor pattern resultant of different concentrations of pregabalin and GABA-caproic for adult zebrafish.** The graphs show the total distance traveled (A) and time immobile (B) during 6 min. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering  $p \leq 0.05$  as significant. No statistical differences were found.

**Fig. 3. Endpoint analysis for the assessment of anxiolytic-like behavior.** Graphs show time spent at the top (A), middle (B) and bottom (C) areas. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering  $p \leq 0.05$  as significant. No statistical differences were found.

**Fig. 4. Entries to the top area.** Graph shows the number of entries to the top zone of novel tank (A). Data were analyzed Kruskal Wallis test, considering  $p \leq 0.05$  as significant. No statistical differences were found.

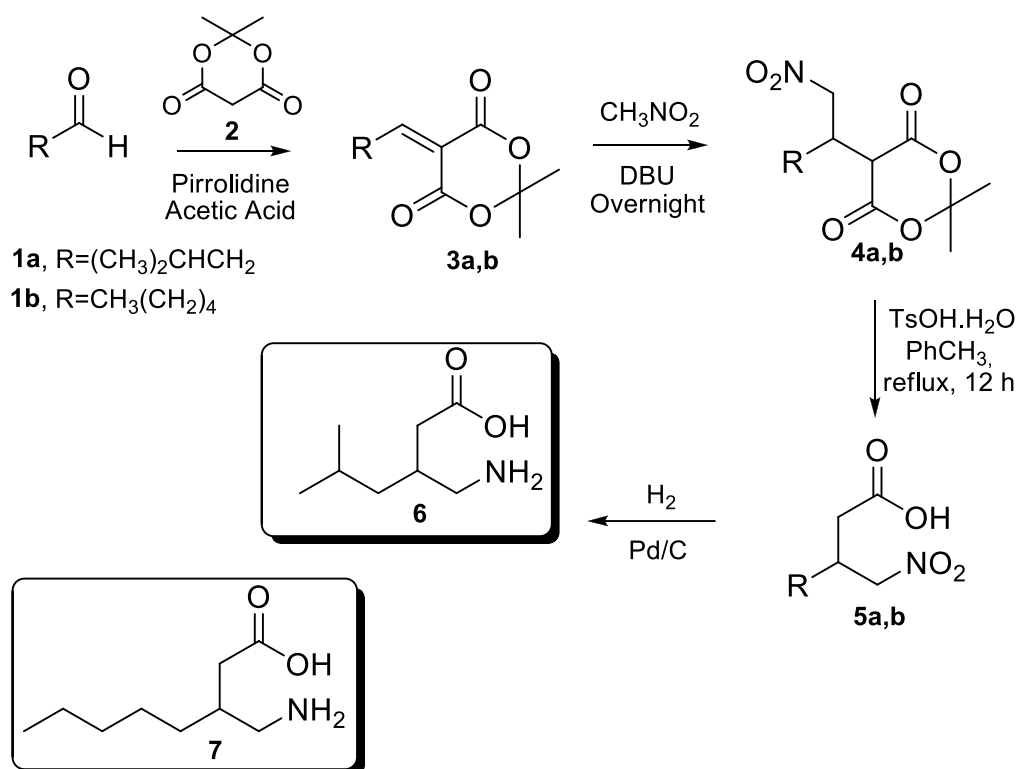
**Fig. 5. Latency to enter the top area.** Graph show time (s) to first entry to the top area (A). Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering  $p \leq 0.05$  as significant. No statistical differences were found.

**Fig. 6. Behavioral profile of PTZ-induced seizures in adult zebrafish.** Seizure scores curve (the highest score reached in each interval) during total 20 min for control (A), pregabalin 20 mM (B), pregabalin 60 mM (C), GABA-caproic 60 mM (D). Seizure intensity was evaluated by the area under curve observed for animal in each group in three separated moments: 0–150 s (E), 150–300 s (F), and 300–1200 s (G). Seizure scores are represented as median  $\pm$  interquartile range. The area under the curve (AUC) is represented as mean  $\pm$  S.E.M and analyzed by the one-way ANOVA followed by the Bonferroni's test as post hoc. \* indicates statistical differences between control group

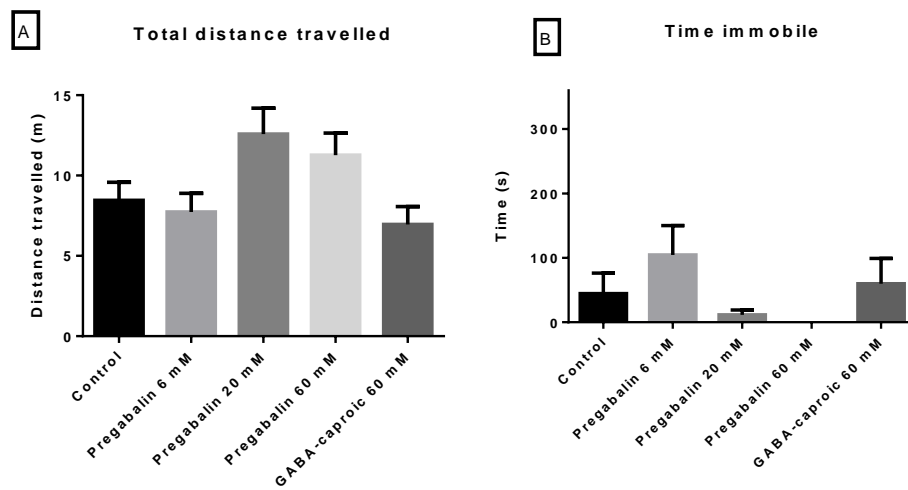
(black) and the other groups (gray bars). In all analyses, the significance level was taken as  $p \leq 0.05$ .

**Fig. 7. Cumulative score frequency.** Cumulative curve of animals (%) which reached scores (0 to 5) during 1200 s observed for each treatment: control (A), pregabalin 20 mM (B), pregabalin 60 mM (C), GABA-caproic 60 mM (D).

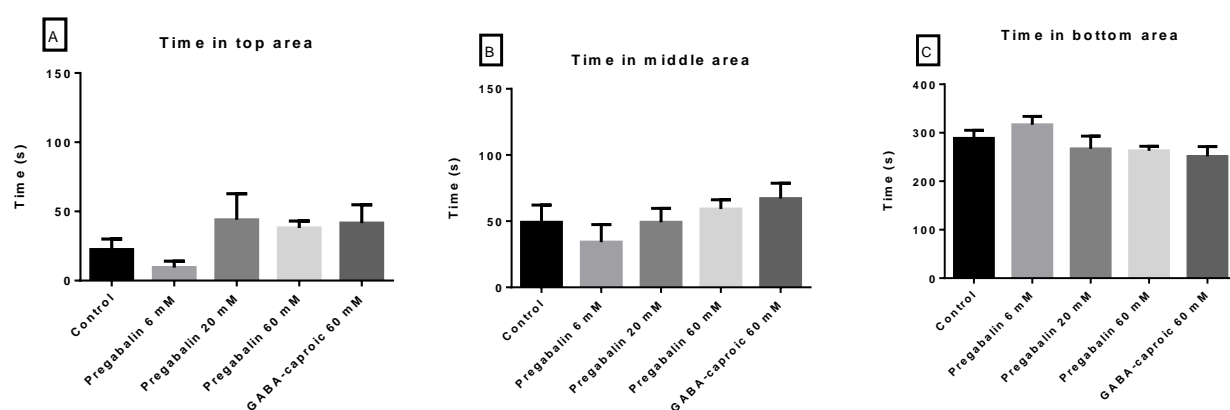
**Fig. 8. Latency to score 4 onset (A).** Latency for each group (control, pregabalin 20, pregabalin 60, GABA-caproic 60 mM) are represented as mean  $\pm$  S.E.M and analyzed by one-way ANOVA followed by Bonferroni's test as post-hoc. Distinct letters indicate statistical differences between control group (black) and the other groups (gray bars). In all analyses, the significance level was taken as  $p \leq 0.05$ .



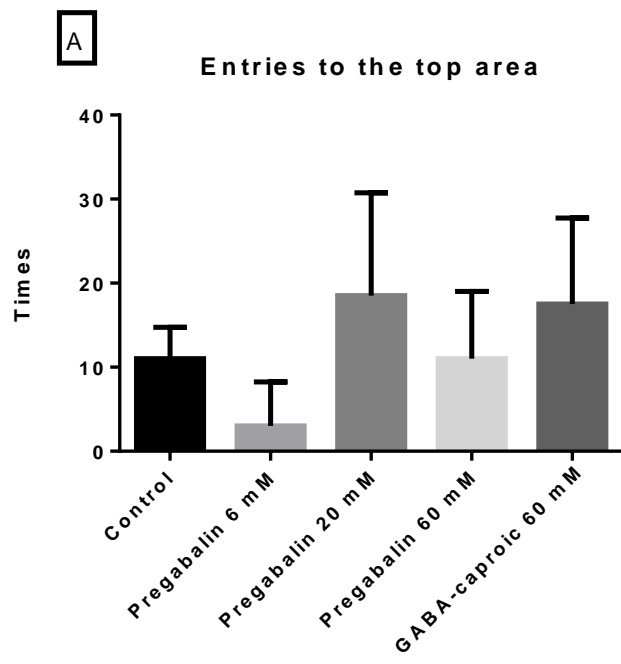
**Fig. 1.** Synthesis of tested compounds Pregabalin (**6**) and GABA-Caproic.



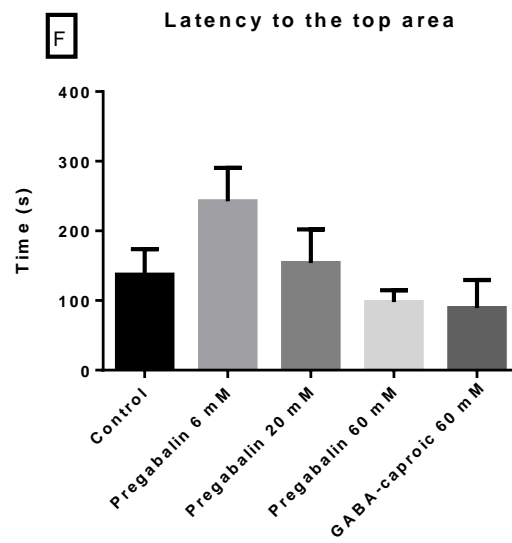
**Fig. 2. Endpoint behaviors used to assess locomotor pattern resultant of different concentrations of pregabalin and GABA-caproic for adult zebrafish.**



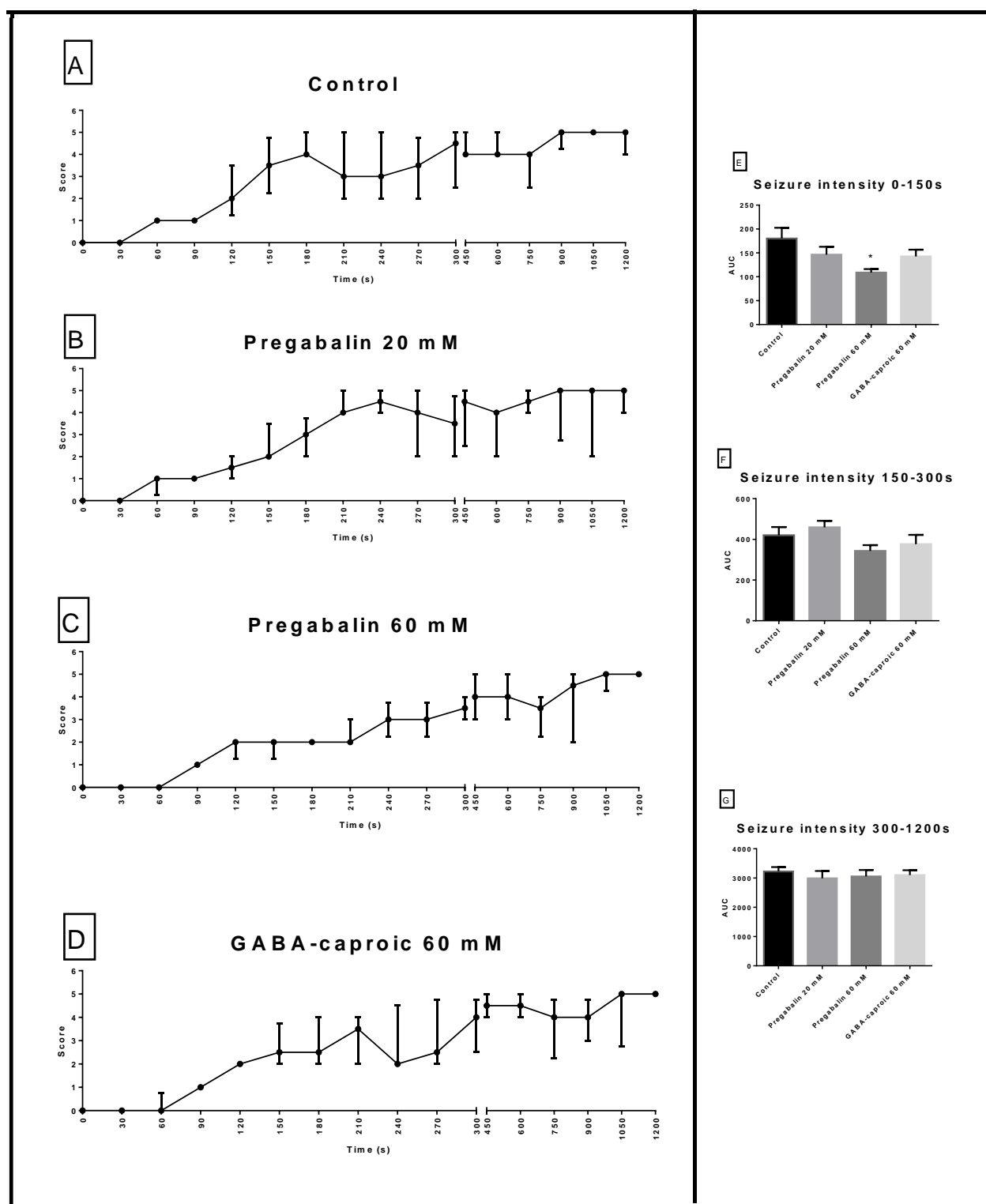
**Fig. 3.**Endpoint analysis for the assessment of anxiolytic-like behavior.



**Fig. 4. Entries to the top area.**



**Fig. 5. Latency to enter the top area.**



**Fig. 6.** Behavioral profile of PTZ-induced seizures in adult zebrafish.



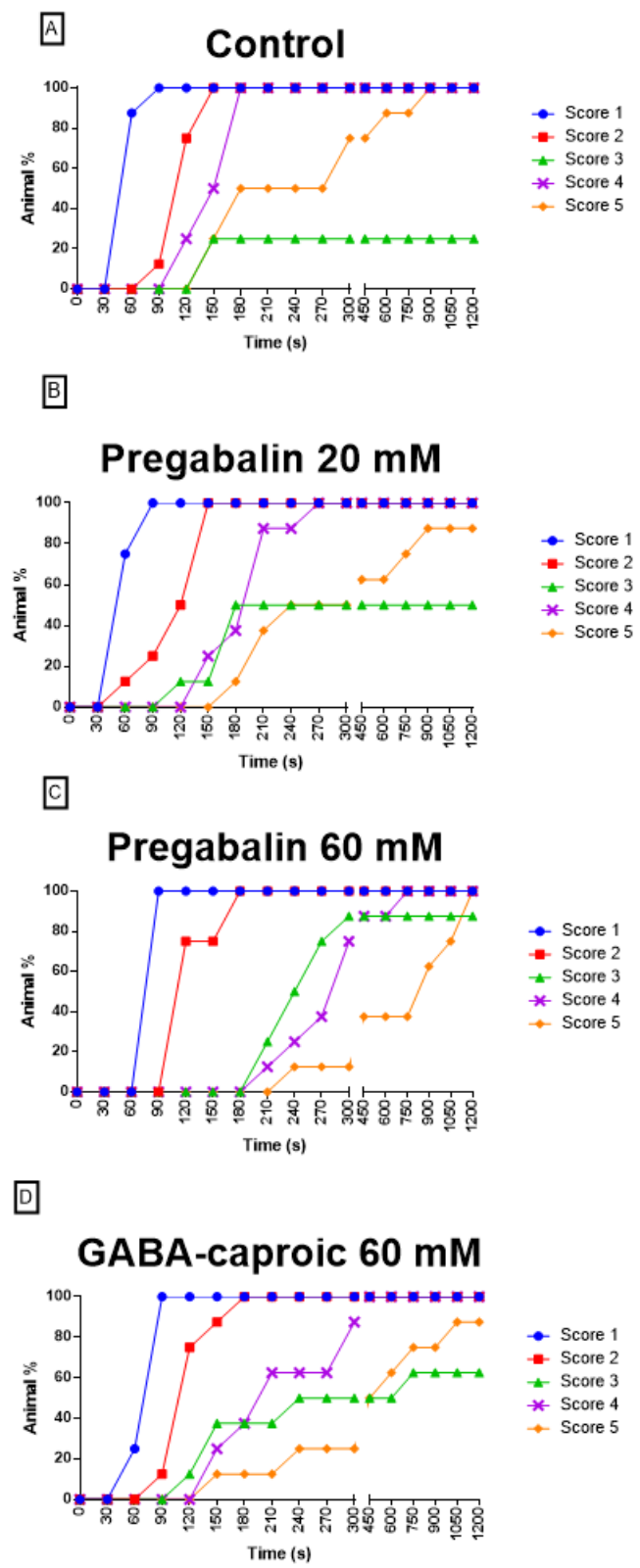
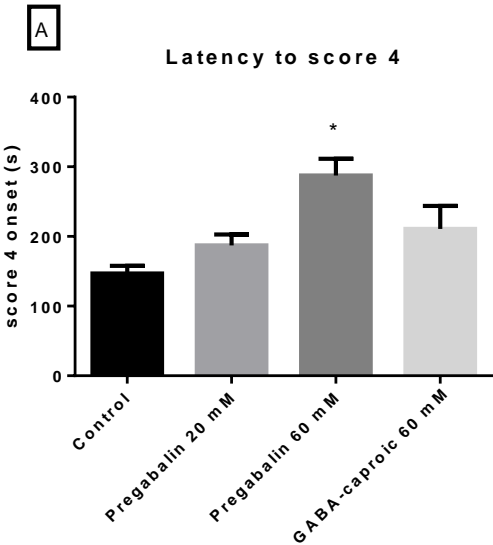


Fig. 7. Cumulative score frequency.



**Fig. 8. Latency to score 4 onset (A).**

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## 6. Conclusões gerais

Concluindo, tanto os animais pré-tratado com pregabalina na concentração de 20 e 60 mM quanto seu derivado sintético GABA-capróico na concentração de 60 mM tendem a aumentar o seu perfil locomotor e exploratório quando submetidos ao teste *novel tank*. Segundo dados da literatura, isso representaria uma tendencia das moléculas apresentarem efeito ansiolítico no modelo animal de peixe-zebra adulto. Efeitos colaterais relacionados à locomoção dos animais não foram encontrados nessas concentrações. A pregabalin demonstrou efeito anticonvulsivo na concentração de 60 mM frente a crises epiléticas induzidas por pentilenotetrazol em modelo de peixe-zebra adulto. Apregabalina 20 mM e o GABA-capróico 60 mM foram capazes de diminuir a intensidade das crises epiléticas em níveis menores que a pregabalina 60 mM, porém, ainda exibindo resultados de intensidade de crises epiléticas menores que o grupo controle.

## 7. Perspectivas

- Aumentar o número de animais por grupo no experimento de *open tank*, a fim de definir um padrão mais claro sobre a exploração dos animais no aparato comportamental;
- Aumentar o número de animais por grupo no experimento de indução de crises epiléticas;
- Calcular o tempo total de duração de estágios 4 (crises clônicas) e 5 (perda de postura, queda ao fundo do aquário e convulsões predominantemente tônicas) em todos os grupos experimentais;
- Testar outras concentrações de GABA-capróico;
- Realizar um teste de preferência animal pelo claro/escuro. Também seria interessante realizar novos experimentos com uma concentração elevada do composto derivado da pregabalina;
- Realizar testes para solubilizar dos demais análogos sintetizados, tendo em vista que a alta hidrofobicidade das cadeias graxas desses compostos dificultam sua solubilidade, mesmo na concentração máxima utilizada pela literatura de DMSO (1%).

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## 9. Anexo 1- Normas de publicação da revista

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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Results should be clear and concise.

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Strunk Jr., W., White, E.B., 2000. *The Elements of Style*, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), *Introduction to the Electronic Age*. E-Publishing Inc., New York, pp. 281–304.

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